HETEROCYCLES, Vol. 82, No. 1, 2010, pp. 273 - 279. © The Japan Institute of Heterocyclic Chemistry
Received, 31st May, 2010, Accepted, 221st June, 2010, Published online, 22nd June, 2010
DOI: 10.3987/COM-10-S(E)41

# AMIDATION OF HYALURONIC ACID WITH A METHOTREXATE-DERIVED AMINE: OPTIMIZATION OF THE KEY REACTION FOR THE SYNTHESIS OF A CANDIDATE DRUG FOR THE TREATMENT OF OSTEOARTHRITIS

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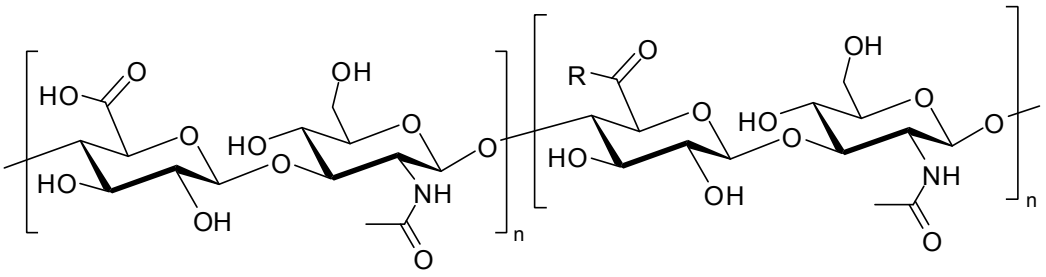
**Abstract** – Optimal amidation conditions between hyaluronic acid (HA) and a methotrexate (MTX)-derived amine that gave rise to a HA-MTX conjugate having a molecular weight of ~2000 kDa have been established based on a model experiment using a tryptophan-derived amine in aqueous THF. This method is robust and provides a conjugate with properties of primary importance for efficacy—MW and amine binding ratio—that satisfy the criteria for a HA-MTX conjugate to be a good candidate.

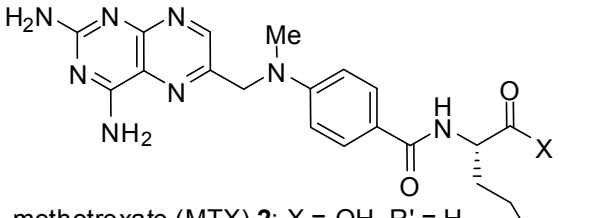
Recently, we synthesized a series of the amide conjugates of hyaluronic acid (HA) **1** and the primary amine derivatives of methotrexate (MTX) **2** intending to develop a safe and effective drug for the treatment of osteoarthritis (OA). HA **1**, a natural polysaccharide of disaccharides composed of D-glucuronic acid and D-*N*-acetylglycosamine linked together via alternating β-1,4- and β-1,3-glucosidic bonds ranging in molecular weight (MW) from 500 to 6000 kDa, is widely used for treatment of OA[1] owing to its synovial fluid viscoelasticity and lubricant effect by knee injection. MTX **2**, an unnatural

Dedicated to Professor Dr. Albert Eschenmoser on the occasion of his 85th birthday

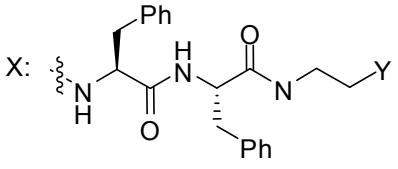
analog of folic acid, is used for treatment of synovial inflammation of rheumatoid arthritis[2] by oral application despite adverse events. Hence, we presumed a conjugation of HA **1** and MTX **2** with an appropriate application to be a clinically safer and much more useful drug than the agents used separately. Among the conjugates examined, we found that knee injection of conjugate **3** (Figure 1) having a peptide spacer with a MW of more than 1800 kDa generated from the MTX-derived primary amine **4** exhibited significant inhibition of knee swelling in antigen-induced arthritis rat and thus supported the rationale for the design of our drug.[3,4] The efficacy of conjugate **3** was almost the same as the oral treatment of MTX and weekly knee injection of conjugate **3** at the significantly reduced dosage of 1/150 that of oral MTX treatment would reduce the risks of systemic side effects. We also found that conjugate **3** having a MW of less than 800 kDa exhibited much lower effect than required for practical use; therefore, as a property of conjugate **3** for medicinal use, it is critically important to maintain a MW of more than 1800 kDa during synthesis. At that time, we could not determine the optimal conditions for consistent production of conjugate **3** with a MW of around 2000 kDa because, depending on the conditions, the amidation process



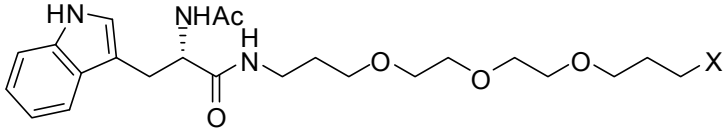
hyaluronic acid **1**: R = OH
drug candidate, conjugate **3**: R = **b**
model amide **7**: R = **d**



methotrexate (MTX) **2**: X = OH, R' = H
MTX-derived amine **4**: X = **a**, R' = Et
**b**: X = **c**, R' = H



**a**: Y = $NH_2$
**c**: Y = NH-



tryptophan-derived amine **6**: X = $NH_2$
**d**: X = NH-

**Figure 1**. Structure of hyaluronic acid **1**, MTX **2**, conjugate **3**, MTX-derived amine **4**, tryptophan-derived amine **6** and amide **7**

resulted in MWs ranging from 800 to 2000 kDa. Herein, we describe our efforts to establish conditions optimal for the key amidation step to consistently afford conjugate **3** having a MW of around 2000 kDa by the reaction between HA **1** and MTX-derived amine **4** (Figure 1).

To date, there have been some precedents from amidation between HA **1** and a primary amine in the presence of an activating agent such as benzotriazole-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP)[5] or 2-chloro-4,6-dimethoxy-1,3,5-triazone (CDMT).[6] However, the MWs of the corresponding amide products reported were at the most 540 kDa even though the MW of the HA **1** is 1200 kDa, indicating that degradation of an intervening activated intermediate such as **5** is by competitive β-elimination during the amidation sequence[6] (Figure 2). Most likely, a similar degradation often occurred in our experiments that resulted in conjugates with lower MWs. We presumed the difficulty to form conjugate **3** with a constant MW was due to the insolubility of HA **1** in most organic solvents which prevented the generation of activated intermediate **5** and its reaction with the amine; and any activated intermediate **5** that was generated was degraded by β-elimination into smaller HA derivatives which in turn resulted in conjugate **3** with a low MW.

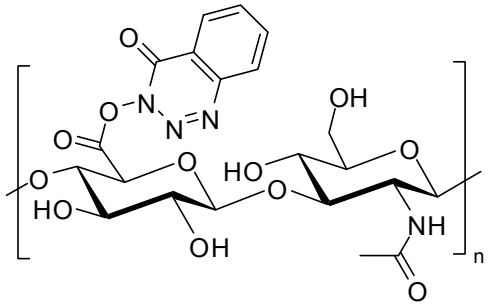



**Figure 2.** Active intermediate **5** (with HOOBt)

We, therefore, sought a solvent appropriate for the amidation of HA **1** using primary amine **6** having tryptophan chromophore as the model to allow estimation of the binding ratio on the basis of UV absorption. An aqueous solution seemed appropriate for the amidation of the HA **1** sodium salt as water has been reported to result in a solution of 10 mg/mL[7] of the salt. Actually, water homogeneously dissolved the HA **1** sodium salt up to 20 mg/mL but resulted in a heavy viscose fluid unable to be properly stirred, probably owing to its high MW.[8] However, we found that the addition of some THF allowed easy stirring at room temperature even though it formed a suspension. We could not find a solvent system that completely dissolved the suspension into a homogeneous solution and so we attempted amidation of the HA **1** sodium salt having a MW of 2170 kDa with amine **6** in an aqueous suspension of THF. The molecular weights of the products were analyzed by gel permeation chromatography (GPC).

At first, three activating agents known to promote the reaction of amidation,[7–10] 1-hydroxybenzotriazole (HOBt), 1-hydroxy-7-azabenzotriazole (HOAt), and 3,4-dihydro-3-hydroxy-1,2,3-benzotriazin-4(3*H*)-one (HOOBt) were compared in an aqueous THF solution (THF:water, 1:9) containing 10 mg/mL of sodium salt of HA **1** (MW of 2170 kDa) and 30 mol% (equivalent to the disaccharide unit of HA **1**) of tryptophan-containing amine **6**.[11] When an additive was not present, virtually no reaction occurred with an equimolar amount of *N*-ethyl-*N'*-3-dimethylaminopropylcarbodiimide hydrochloride (EDC)[9,10] and the HA **1** and amine **6** were unchanged (Table 1, Entry 1). On the other hand, when an equimolar amount to **6** of each additive was present, the reaction gave amide **7** (Entries 2–4). However, in every case both the

**Table 1.** The effects of water contents and reagents amounts for amidated HA conjugate **7** synthesis[a]

| Entry | $H_2O$:THF[b] | EDC (eq)[c] | **6** (eq)[c] | Additive (eq)[c] | Binding ratio (%)[d] | MW (kDa)[e] |
|---|---|---|---|---|---|---|
| 1 | 1:9 | 0.3 | 0.3 | none | 0 | 2170 |
| 2 | 1:9 | 0.3 | 0.3 | 0.3: HOOBt | 14.9 | 740 |
| 3 | 1:9 | 0.3 | 0.3 | 0.3: HOAt | 8.8 | 840 |
| 4 | 1:9 | 0.3 | 0.3 | 0.3: HOBt | 4.9 | 780 |
| 5 | 1:9 | 0.1 | 0.3 | 0.3: HOOBt | 7.3 | 1100 |
| 6 | 1:4 | 0.1 | 0.3 | 0.3: HOOBt | 7.7 | 1220 |
| 7 | 1:1 | 0.1 | 0.3 | 0.3: HOOBt | 8.8 | 2220 |
| 8 | 1:1 | 0.8 | 0.3 | 0.3: HOOBt | 20.9 | 1060 |
| 9 | 1:1 | 0.2 | 0.3 | 0.3: HOOBt | 15 | 1370 |
| 10 | 1:1 | 0.1 | 0.2 | 0.2: HOOBt | 5.1 | 2270 |
| 11 | 1:1 | 0.1 | 0.1 | 0.1: HOOBt | 4.1 | 2310 |

[a] Experimental conditions: A mixture of sodium salt of HA **1**, EDC, amine **6**, additive, $H_2O$ and THF was stirred for 20 h at 4 °C.

[b] Solvent volume of 100 mL/g (for HA).

[c] Equivalent values were based on the value for the carboxylic acid group of HA.

[d] The binding ratio of Ac-Trp-PEG amine **6** was calculated by measuring HPLC UV absorption (254 nm).

[e] Molecular weights were determined by gel permeation chromatography.

binding ratio and the MW of the resulting amide **7** were less than satisfactory and accompanied by fragmented HA, indicating that degradation of the active intermediate **5** had occurred. In order to minimize undesirable degradation, we increased the ratio of water in the THF and further examined the amidation reaction in the presence of HOOBt, which we considered to be the best of the additive tested.[7–10] All reactions produced amide **7**, even in 50% aqueous THF. However, the only products that satisfied our criteria showed molar ratios of EDC that did not exceed those of amine **6** and the additive (Entries 7, 10 and 11). Thus, an amide **7** with a satisfactory MW of around 2200 kDa could be obtained with 3 equimolar amounts of amide **6** even though the binding ratio was somewhat unsatisfactory when equivalent amounts of reagents were used (Entry 11). Particularly interesting is that the reactions in 10% and 20% aqueous THF produced similar amides **7** of MW 1100–1200 kDa (Entries 5, 6) but the same reaction in 50% aqueous THF generated amide **7** of MW 2220 kDa (Entry 7). Moreover, the amides with a MW that did not fulfill our requirement were produced in the presence of more than 0.2 molar EDC (Entries 8, 9) despite having high binding ratios. Amide **7** with a higher molecular weight could be obtained by using an equimolar of amine **6** and an additive in 50% aqueous THF (Entries 7, 10 and 11). We concluded that amidation of HA **1** with amine **6** should be carried out in 50% aqueous THF containing 10 mg/mL of HA **1** sodium salt in the presence of 0.1 molar EDC to minimize competitive β-elimination leading to lower MW amide **7** (Entries 7, 10, and 11). Taking into account the MW and the binding ratio observed in this study as well as amount of the additive used, we concluded that the conditions of Entry 11 are suitable for the synthesis of our drug candidate, conjugate **3**.

Having established the optimal conditions using tryptophan-derived amine **6** as the model, we next carried out amidation between HA **1** and MTX-derived primary amine **4**[12] to obtain **3**, our target drug candidate, with a MW of more than 1800 kDa.[4] Thus, to a stirred suspension of HA **1** sodium salt (MW of ~2300 kDa, 2.0 g) in THF (40 mL) was added a solution of HOOBt (0.1 eq, 0.50 mmol) and MTX-derived amine **4** (0.025 eq, 0.125 mmol) in 50% aqueous THF (80 mL), followed by a solution of tris[2-(2-methoxyethoxy)ethyl]amine (0.075 eq, 0.38 mmol) in 50% aqueous THF (40 mL) at 5 ºC. Stirring was continued at the same temperature for 30 min and to this mixture was then added a solution of EDC (0.1 eq, 0.50 mmol) in water (40 mL) and stirring was continued for 20 h at the same temperature. The reaction mixture was sequentially treated with 0.09 N NaOH (880 mL), 1 N HCl (80 mL), 20 wt% NaCl (140 mL) and ethanol (2400 mL) to give the product as a precipitate which was collected by centrifugation. The collected product was dissolved in 3 wt% NaCl solution (800 mL) and treated with ethanol (1600 mL) to give the product as a precipitate which was collected by centrifugation. The collected product was dissolved in water (1000 mL) and further purified by filtration using a 0.45-μm filter (Stervex HV; Millipore) to give an aqueous solution of target conjugate **3** having a MW of 2110

kDa and a binding ratio of 2.2%. The molecular weight and binding ratio of the conjugate **3** of HA **1** and amine **4** generated under the conditions optimized according to our examination was found to be somewhat less satisfactory than we initially expected but was reproducible and the resultant **3** exhibited satisfactory efficacy in the treatment of the OA in the model.[2]

In conclusion, we have established optimal conditions for the amidation between HA **1** and MTX-derived amine **4** and generated amide conjugate **3** with a molecular weight of ~2000 kDa, a promising drug candidate for the treatment of OA with no appreciable degradation of the HA molecule.

**ACKNOWLEDGEMENTS**

The authors thank Professor Kunio Ogasawara for his helpful suggestions concerning this study. We also thank Ms. Frances Ford for proof reading the manuscript.

**REFERENCES AND NOTES**

1. M. Pagnano and G. Westrich, *Osteoarthritis Cartilage*, 2005, **13**, 751.
2. J. Swierkot and J. Szechinski, *Pharmacological Reports*, 2006, **58**, 473.
3. A. Homma, H. Sato, A. Okamachi, T. Emura, T. Ishizawa, T. Kato, T. Matsuura, S. Sato, T. Tamura, Y. Higuchi, T. Watanabe, H. Kitamura, K. Asanuma, T. Yamazaki, M. Ikemi, H. Kitagawa, T. Morikawa, H. Ikeya, K. Maeda, K. Takahashi, K. Nohmi, N. Izutani, M. Kanda, and R. Suzuki, *Bioorg. Med. Chem.*, 2009, **17**, 4647.
4. A. Homma, H. Sato, T. Tamura, A. Okamachi, T. Emura, T. Ishizawa, T. Kato, T. Matsuura, S. Sato, Y. Higuchi, T. Watanabe, H. Kitamura, K. Asanuma, T. Yamazaki, M. Ikemi, H. Kitagawa, T. Morikawa, H. Ikeya, K. Maeda, K. Takahashi, K. Nohmi, N. Izutani, M. Kanda, and R. Suzuki, *Bioorg. Med. Chem.*, 2010, **18**, 1062.
5. E. J. Oh, K. Park, J. S. Choi, C. K. Joo, and S. K. Hahn, *Biomaterials*, 2009, **30**, 6026.
6. K. Bergman, C. Elvingson, J. Hilborn, G. Svensk, and T. Bowden, *Biomacromolecules*, 2007, **8**, 2190.
7. Y. Tokita and A. Okamoto, *Europian Polymer Journal*, 1996, **32**, 1011.
8. Y. Kobayashi, A. Okamoto, and K. Nishinari, *Biorheology*, 1994, **31**, 235.
9. J. W. Kuo, D. A. Swann, and G. D. Prestwich, *Bioconjug. Chem.*, 1991, **2**, 232.
10. T. Pouyani, J. W. Kuo, G. S. Harbison, and G. D. Prestwich, *J. Am. Chem. Soc.*, 1992, **114**, 5972.
11. Tryptophan-derived primary amine **6** was prepared employing a standard procedure from commercially available diamine, *N*-*t*-butoxycarbonyl-4,7,10-trioxa-1,13-tridecanediamine, in a sequence of three steps for an overall yield of 37% involving monoprotection, amidation with

acetyltryptophan and deprotection.

12. MTX-derived primary amine **4** was prepared from *N*-Boc-ethylenediamine in four steps for an overall yield of 55% involving coupling with the tripeptide, Cbz-Glu(OEt)PhePhe-OH, deprotection, coupling with 4-[*N*-(2,4-diamino-6-pteridinylmethyl)-*N*-methylamino benzoic acid and deprotection.